Based on an oral restriction requirement and applicants' oral election, claims 1-4 and 11-26 were withdrawn from further consideration as being directed to **patentably distinct** non-elected subject matter, and only claims 5-10 were examined. Applicants hereby respectfully affirm the election of claims 5-10, which have now been expanded to claims 5-10 and 27-30. However, applicants respectfully request that the restriction requirement as applied to claims 11-26 be withdrawn (applicants accept the PTO ruling that claims 1-4 are patentably distinct from the elected method, and *vice versa*).

If the examiner will again carefully consider the non-elected claims 11-26, it will be evident that claim 11 is clearly directed to the result or product of the method of claim 5. Under the circumstances of such a direct relationship, the restriction requirement should be withdrawn, and claims 11-26 should be examined, and such is respectfully requested.

Applicants' abstract has been criticized. Accordingly, such abstract has now been cancelled and a new abstract is attached hereto. Its approval and entry are respectfully requested.

Claim 5 has been objected to because the examiner considers that the term "active-oxygen-eliminating" is lacking in clarity. This objection is respectfully traversed.

The examiner's attention is invited to applicants' disclosure at page 2, lines 11-15, of the specification, from

which it is clear that the term "active-oxygen-eliminating" is a broader term than "antioxidant" which is a sub-genus of "active-oxygen-eliminating". Enclosed please find copies of Bull. Chem. Soc. Jpn. 65:1447-1453 (1991) and JEOL NEWS, 23A(1):7-9 (1987). From these publications, it should be apparent what is meant by the term "active-oxygen". "Active-oxygen" includes superoxide anion radical, hydroxyl radical, hydrogen peroxidase, singlet oxygen, etc. Applicants believe that the term "active-oxygen-eliminating" is a common technical term and that it should not be necessary to replace the term with "antioxidant".

Withdrawal of the objection is respectfully requested.

Claims 5-10 have been rejected under the second paragraph of §112. The rejection is respectfully traversed, especially insofar as it might presently be deemed to apply.

It is believed that most if not all of the examiner's criticisms have been addressed in the amendments presented above, to thus moot or obviate the rejection. Such amendments are made only made to better particularly point out and distinctly claim the invention, not to overcome any prior art. No limitations have been added and none are intended.

As regards the expression "in an aqueous system", claim 5 is intended to be generic. Either the plant substance may initially be in an aqueous system, such as a juicy form (e.g. see the top paragraph on page 6 of applicants' specification) in which case the inhibitory agent may be applied

as either a liquid or a solid, or the plant substance may be initially solid in which case the inhibitory agent is applied in an aqueous system.

The criticism of claim 6 is not understood. Claim 6 clearly says that the plant substance (recited in claim 5) is a plant edible substance and/or a plant antioxidant. This is the material to which the inhibitory agent of the present invention is added.

The improper claim dependency of claim 7 has now been corrected.

The criticism of claim 9 is not understood. Claim 5 previously depended from claim 1 which recited the presence of trehalose, and therefore "said trehalose" as recited in claim 9 was (and still is) proper. As regards "effective ingredient", the examiner's helpful suggestion has been adopted (although applicants do not understand why the examiner considers the presence of such terminology to render claim 9 indefinite).

The examiner's helpful suggestion has been adopted for claim 10.

Withdrawal of the rejection is respectfully requested.[1]

New claims 27-30 have been added, and support for these claims is believed to be quite clear (for example, see original claims 1-4 for new claims 27 and 28, and the last two

[1] Applicants understand that the claims are otherwise deemed by the PTO to be in full compliance with §112, and applicants are proceeding in reliance thereof.

lines of the first paragraph on page 5 of applicants' specification for new claims 29 and 30). These claims are patentable for the same reasons as claims 5-10, as pointed out below.

Claims 5-10 have been rejected as obvious under §103 from Maruta et al USP 5,472,863 ("Maruta"). However, the body of the rejection also refers to "Kubota". Accordingly, the rejection is not entirely clear to applicants. To the extent that applicants do understand the rejection, it is respectfully traversed.

It is respectfully pointed out that Maruta teaches nothing about active-oxygen-eliminating activity of a plant substance. Maruta discloses only that trehalose may be used as a stabilizer for biologically active substances. The other parts of the grounds for rejection are based, respectfully, on pure speculation. It is believed that even a person of more than ordinary skill in the art would never have been motivated to use trehalose as an effective ingredient in a method of inhibiting the decrease of active-oxygen-eliminating activity.

The rejection is based on §103, and therefore anticipation is not the issue. The question to be answered is whether one of ordinary skill in the art at the time the present invention was made would have been motivated by anything in the prior art (Maruta alone or Maruta in view of Kubota) to use trehalose to inhibit the decrease of active-oxygen-eliminating activity. As there is not the remotest hint in the prior art

that trehalose would be capable of providing such an inhibitory effect, it cannot be validly said that it would have been obvious to even try (let alone have a reasonable expectation of success) trehalose for such an objective.

The rejection states that it would have been obvious to one of ordinary skill in the art to have incorporated trehalose into edible plant substances, as taught by Kubota, where the motivation would have been to enhance the quality and "healthfulness" of the finished product. Respectfully, applicants consider this reasoning to be illogical, and at best based on applicants' specification. Kubota suggests nothing about the claimed invention, as well as active-oxygen-eliminating activity of a plant substance. With respect, such reasoning set forth in the rejection overlooks the objective and the results achieved. It could not have been predicted or foreseen from the citations that trehalose would produce the results achieved, and therefore there would have been no reason or motivation for using trehalose for that purpose.

As regards enhancing the quality and "healthfulness" of a particular food product, it can be said that the incorporation of any healthy ingredient into another food product, regardless of the unexpected results it provides, would never be patentable for that reason. The point to be emphasized is that applicants have discovered a property of trehalose which was never previously known, and the claimed method is directed to the utilization of that previously unknown property to obtain an unexpected result, namely the inhibition of the decrease or

reduction of active-oxygen-eliminating activity of a plant substance which possesses that activity.

The citations do not make applicants' process obvious. Consequently, applicants request that the rejection be withdrawn.

Claims 5-10 have also been rejected as obvious under §103 from Hersh in view of Kubota JP 07-213283 ("Kubota") and further in view of the Carpenter et al publication ("Carpenter"). This rejection is respectfully traversed.

The rejection states that Hersh discloses a method of preventing redox cycling reactions in order to protect an antioxidant, and agrees that Hersh does not teach trehalose nor a plant substance comprising antioxidant activity. It seems to applicants that what is so stated in the rejection indicates that Hersh has nothing to do with the claimed invention.

Neither Kubota nor Carpenter teaches any active-oxygen-eliminating activity of a plant substance, nor do they teach the use of trehalose as an effective ingredient in a method for inhibiting the decrease of active-oxygen-eliminating activity. Under these circumstances, applicants' invention could not be reached even if it were obvious to attempt to combine the three disparate citations, and applicants cannot see how any possible combination of such references (even if obvious, respectfully denied) could lead to or result in the present invention.

Moreover, even if the combination were obvious and did lead toward the present invention (both respectfully denied by applicants), the present invention would still be unobvious for the same reasons pointed out above with respect to the rejection based on Maruta. Thus, applicants discovered a previously unrecognized activity, characteristic or ability of trehalose, and the claimed method is directed to the utilization of that previously unrecognized activity. Because the activity was unrecognized, the results of applicants' claimed method are inherently non-obvious.

Withdrawal of the rejection is in order and is respectfully requested.

The prior art documents made of record and not relied upon have been noted, along with the implication that such documents are not deemed sufficiently pertinent by the PTO to warrant their application against any of applicants' claims.

Favorable reconsideration and allowance are earnestly solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By _____
Sheridan Neimark
Registration No. 20,520

SN:rd
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
itt3\sn\aga6.amd

Spin-Trapping Studies on the Reaction of Iron Complexes with Peroxides and the Effects of Water-Soluble Antioxidants

Masahiro KOHNO,* Masako YAMADA, Keiichi MITSUTA, Yokio MIZUTA, and Toshikazu YOSHIKAWA†
ESR Application Laboratory, Analytical Instruments Division, JEOL Ltd.,
1-2 Musashino 3-Chome, Akishima, Tokyo 196
† First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602
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Using ESR spin-trapping techniques we measured the levels of free radical species generated from six different systems (hypoxanthine–xanthine oxidase, iron(II)–hydrogen peroxide, iron(III)–hydrogen peroxide, iron(II)–*t*-butyl hydroperoxide, iron(III)–*t*-butyl hydroperoxide, and catalase (CAT)–*t*-butyl hydroperoxide). Six types of radicals ($O_2^{\cdot-}$, ·OH, ·H, ·CH_3, $(CH_3)_3CO\cdot$, and $(CH_3)_3COO\cdot$) were detected as spin adducts of spin traps 5,5-dimethylpyrroline 1-oxide (DMPO) or 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS). Quantitative analysis of the levels of generated radicals by means of an ESR instrument also presents important information regarding the reduction of peroxides [hydrogen peroxide (H_2O_2) or *t*-butyl hydroperoxide (ROOH)] by iron(II) or iron(III) as well as catalase. In addition, the scavenging potencies of different water-soluble antioxidants such as L-ascorbic, D-isoascorbic, gallic, sorbic, and protocatechuic acids were evaluated in terms of their ability to reduce the peaks of spin adducts.

Active oxygens such as superoxide anion radical ($O_2^{\cdot-}$, ·OOH), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) have been implicated as being major damaging species in pathology and have been widely investigated.[1] These compounds react with the lipids of membranes and through a series of reactions generate carbon-centered (R·), alkoxyl (RO·), and then peroxyl (ROO·) radicals, all of which are used as markers of lipid peroxidation and the disruption of cellular homeostasis.[2,3] Also widely explored are studies scavenging phenomena of these active oxygens and free radicals by such antioxidants as vitamins C and E, and glutathione, and by scavengers such as superoxide dismutase (SOD) as well as catalase (CAT).[4-7]

The ESR spin-trapping technique is very useful for stabilizing short-lived free radicals. This technique has therefore been a powerful tool for studying generation mechanisms of free radicals and active oxygens[8-10] as of free radicals.[11-23]

The present study was carried out in order to establish a detection method for such free radicals as $O_2^{\cdot-}$, ·OH, ·H, ·CH_3 (R·), $(CH_3)_3CO\cdot$ (RO·), and $(CH_3)_3COO\cdot$ (ROO·). The six radical-generating systems were: hypoxanthine–xanthine oxidase which generates $O_2^{\cdot-}$ and ·OOH; iron(II)– hydrogen peroxide which gives ·OH; iron(III)–hydrogen peroxide, ·OH; iron(II)–*t*-butyl hydroperoxide and iron(III)–*t*-butyl hydroperoxide, R·, RO·, ROO·, and CAT–*t*-butyl hydroperoxide, RO·. The mechanism in Fenton's or catalase reactions between iron(II), iron(III), or CAT and ROOH or H_2O_2, are also described based on the results obtained from experiments performed under several conditions. Finally, the potencies of water-soluble antioxidants (L-ascorbic acid, D-isoascorbic acid, gallic acid, sorbic acid, and protocatechuic acid) in scavenging $O_2^{\cdot-}$, ·OH, ROO·, and/or RO· are described.

Experimental

Materials. Spin trapping reagents 5,5-dimethylpyrroline 1-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS), were supplied by Mitsui Toatu Chemicals and by Sigma Chemical Co., Ltd., respectively. Diethylenetriamine-*N*,*N*,*N*′,*N*′,*N*″-pentaacetic acid (DETAPAC), used to chelate trace metal impurities, was obtained from Wako Pure Chemical. The following were the sources of different radicals and their corresponding suppliers: sources of superoxide radical [hypoxanthine (HPX) from Sigma Chemical, and xanthine oxidase (XOD) from Boehringer Mannheim, cow milk]; source of hydroxyl radical [iron(II) sulfate heptahydrate (ferrous iron) and iron(III) sulfate *n*-hydrate (ferric iron) from Wako Pure Chemical Ins., Ltd., and hydrogen peroxide (H_2O_2)]; source of methyl, *t*-butoxyl, *t*-butylperoxyl radicals [iron(II) or iron(III) and *t*-butyl hydroperoxide (ROOH) from Nakarai Kagaku Co.]. Antioxidant reagents L-ascorbic acid, D-isoascorbic acid (erythorbic acid), and gallic acid were purchased from Daiichi Pure Chemical, while sorbic acid and protocatechuic acids were obtained from Wako Pure Chemical Ins.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using aqueous quartz flat cell (Inner size 60 mm×10 mm×0.31 mm) with an effective sample volume 160 μl.

Preparation of Samples. All measurements were carried out both in 0.1 M (M=mol dm^{-3}) of PBS (sodium phosphate buffer solution) (pH=7.8) and in pure water at room temperature. Both peroxides, H_2O_2 and ROOH, were used as aqueous solutions. The concentration of the antioxidants used in this experiment was 1.0 mM (1 mM=1.0×10^{-3} mol dm^{-3}). Superoxide radicals were generated from a hypoxanthine xanthine oxidase reaction system under the conditions reported previously.[15,17]

Results and Discussion

Detection and Identification of Radicals [$O_2^{\cdot-}$, ·OH, ·H, R·, RO·, and ROO·]. The ESR spectra of the spin

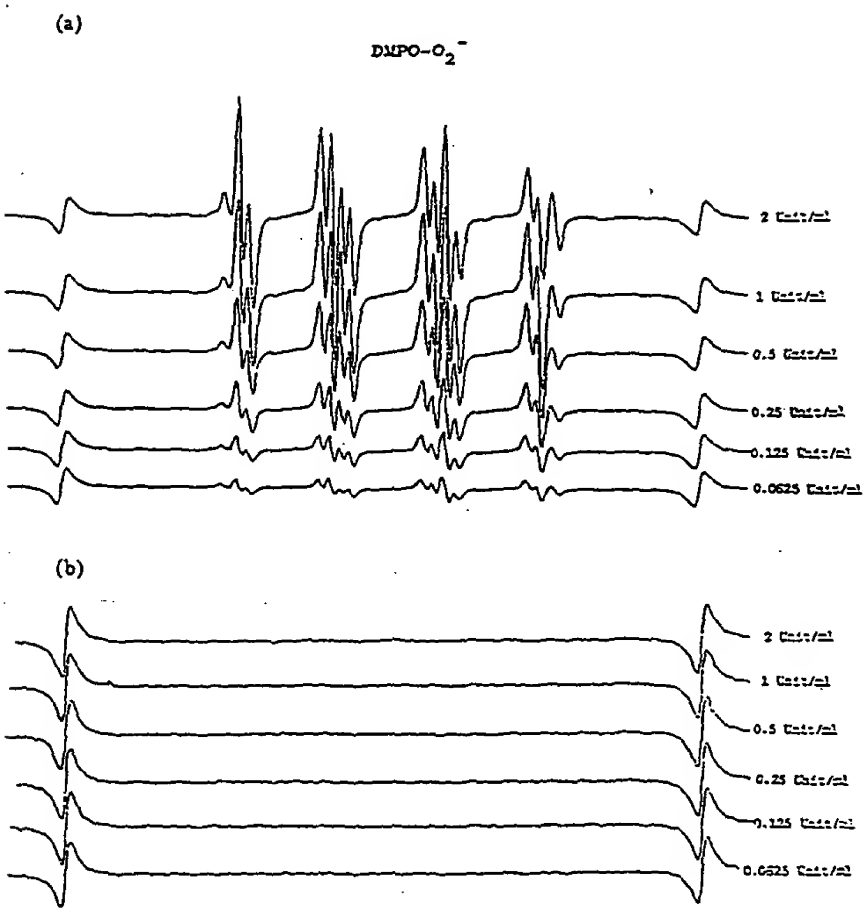

Fig. 1. ESR spectra of spin adducts of $O_2^{\cdot-}$, observed by the use of spin traps DMPO (a) and DBNBS (b). Spectrum (a) recording started 30 s after mixing 50 µl 2 mM HPX, 35 µl 5.5 mM DETAPAC, 15 µl 9.2 M DMPO (a), 50 µl 0.1—0.5 U ml^{-1} XOD, and 50 µl PBS. Spectrum (b) was observed under same conditions but without 15 µl 60 mM DBNBS.

adducts obtained in the hypoxanthine-xanthine oxidase reaction by using two kinds of spin traps, DMPO and DBNBS, are shown in Figs. 1a and 1b. In these figures, the peaks at either ends are of Mn^{2+} in MgO, which is used as an internal standard. The g values of both peaks are 2.0334 and 1.9810 at the resonance frequency of 9450.0 MHz, respectively. The method of measurement and the reaction conditions used in the experiments have been described previously.[10,17] As shown in Fig. 1a, a spin trapped by DMPO increases with the XOD concentration, and the hyperfine coupling constants (hfcc) obtained from the spectra coincide with the values of DMPO-O_2^- reported previously.[8] Using the spin trap DBNBS instead of DMPO, no signals were observed under the same conditions (Fig. 1b). However, the addition of DBNBS (60 mM) reduced the signal intensity of DMPO-O_2^-. From the experimental results for changing the concentration of DBNBS, the value of 50% inhibition($1D_{50}$) of DMPO-O_2^- was measured to be 0.3 mM. The reaction rate constant (k_2) of DBNBS with $O_2^{\cdot-}$ in a hypoxanthine-xanthine odixase reaction were determined to be 3.9×10^2 M^{-1} s^{-1} at pH=7.8 from ID_{50} by treating for competitive reaction.[14] This value is 5.9-times larger than the previous reported value, 6.6×10^1 M^{-1} s^{-1} at pH=7.0, obtained by a pulse radiolysis method.[26] The difference may be due to differences in the pH and generation system of $O_2^{\cdot-}$. The results clearly indicate that both DMPO and DBNBS react with $O_2^{\cdot-}$. However, only DMPO gives a spectrum of the $O_2^{\cdot-}$ spin adduct. No $O_2^{\cdot-}$ spin adduct signals can be detected in the case of DBNBS. It is speculated that nitrones are better than nitroso compounds in detecting $O_2^{\cdot-}$ as a spin adduct.

Figures 2a and 2b show the spectra of the spin adducts obtained using DMPO or DBNBS, respectively, in reactions between 0.1 mM iron(II) chelated by DETAPAC (0.1 mM) and H_2O_2 (1 mM) in pure water.

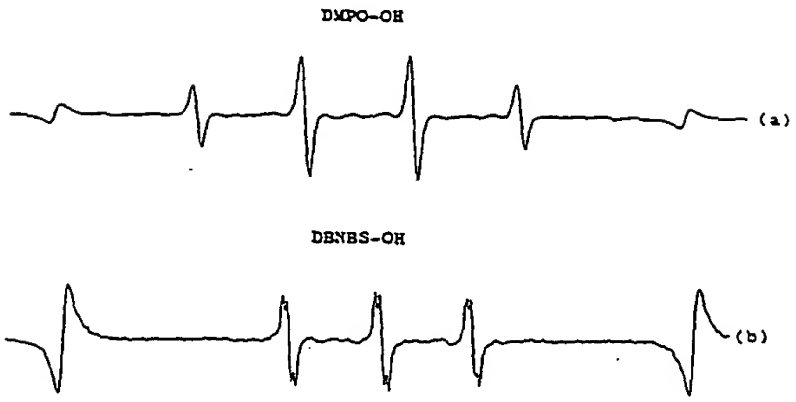

Fig. 2. ESR spectra of spin adducts of ·OH observed by the use of spin traps DMPO (a) and DBNBS (b). Spectra recording started 30 s after mixing 75 μl 0.1 mM iron(II) chelated by 0.1 mM DETAPAC before use [iron(II)-DETAPAC], 75 μl 1 mM H_2O_2, 50 μl 60 mM DMPO and 60 mM DBNBS, in 50 μl pure water.

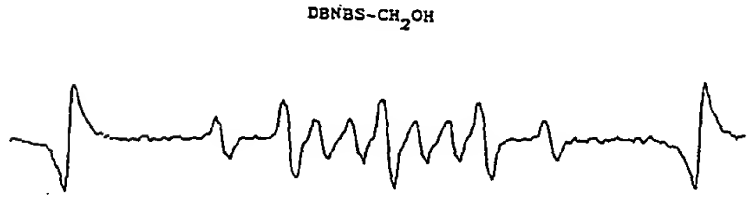

Fig. 3. ESR spectra of spin adducts of $\cdot CH_2OH$ after mixing 75 μl 0.1 mM iron(II)-DETAPAC, 75 μl 1 mM H_2O_2, 15 μl 60 mM DBNBS and 50 μl 1.6 mM methanol.

In the case of DMPO (Fig. 2a), a typical hfcc was obtained as a_N=1.48 and a_{H_β}=1.48 mT, which is the same as the typical value of DMPO-OH.[12] Using iron(III) (0.1 mM) instead of iron(II), less DMPO-OH is generated (Fig. 2b).

The results of the above-mentioned experiments demonstrate that iron(II) is more active than iron(III) in the reduction reaction of H_2O_2. With DBNBS, the hfcc of the signal is analyzed as a_N=1.24 mT and a_H=0.063 mT (Fig. 2b), which coincide with those reported previously.[26,27] However, the previous studies have many uncertainties,[27] since this adduct was assigned to DBNBS-O_2^-. In order to confirm that this radical species was DBNBS-OH, the methanol, which is a specific scavenger of ·OH, was added to the reaction system. Then, the signal decayed with increasing methanol concentration. When 1.6 mM methanol was added, another spin adduct, which is assigned as methanol radical (DBNBS-CH_2OH; a_N= 1.36 mT, a_H=0.91 mT and a_H=0.06 mT),[20,27] is detected as is shown in Fig. 3. In addition, a concomitant increase in the intensity of the DBNBS-OH signal is observed with increasing amount of added H_2O_2 (Fig. 4). Thus, ·OH can be trapped not only by DMPO, but also by DBNBS. The rate constant (k_2) for the reaction of DBNBS with ·OH is determined to be 2.2×10^{10} M^{-1} s^{-1}, as measured from its competitive reaction with DMPO in trapping ·OH radicals. In this treatment, $k_2=3.4\times10^9$ M^{-1} s^{-1} [19] was used as the rate constant for the reaction of DMPO to trap ·OH. From these values, DBNBS is more sensitive than DMPO for detecting ·OH.

In the reaction between iron(II) of 0.1 mM chelated by DETAPAC of 0.1 mM, and H_2O_2 (0.01 mM), two kinds of spin adducts, DMPO-OH and DMPO-H, are observed (Fig. 5). Thus, from these spectra, it is confirmed that at least two types of radicals (·H and ·OH) are generated in the reaction. The concentration of the generated DMPO-OH agrees with that of DMPO-H under this reaction.

Using DMPO, both the reactions between iron(II) or iron(III) and ROOH give three kinds of spin adducts of methyl (R·), *t*-butoxyl (RO·), and *t*-butylperoxyl (ROO·) radicals (Fig. 6). However, when DBNBS was used, only the spin adduct of methyl radical (DBNBS-CH_3: a_N=1.37 mT and a_{CH_3}=1.35 mT and a_H=0.07 mT) was observed (Fig. 7). The reaction between the iron(III) and ROOH give a relatively weak signal of methyl

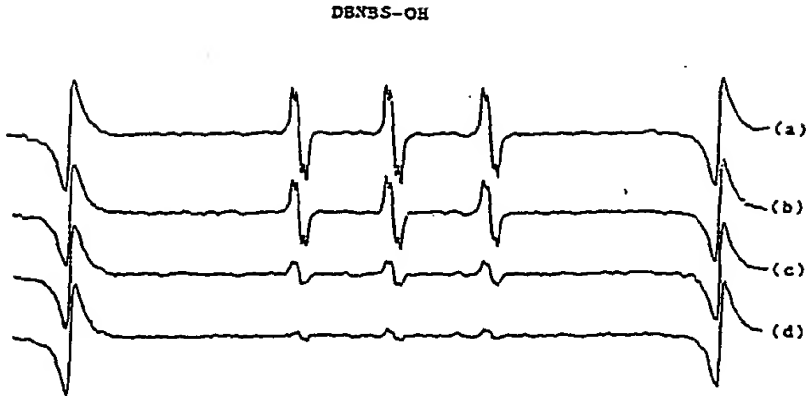

Fig. 4. ESR spectra of spin adducts of ·OH observed by the use of spin trap DBNBS. 75 μl 0.1 mM iron(II)–DETAPAC, 75 μl of (a) 1 mM, (b) 0.5 mM, (c) 0.25 mM and (d) 0.125 mM H_2O_2 concentration, 15 μl 60 mM. DBNBS, in 50 μl pure water.

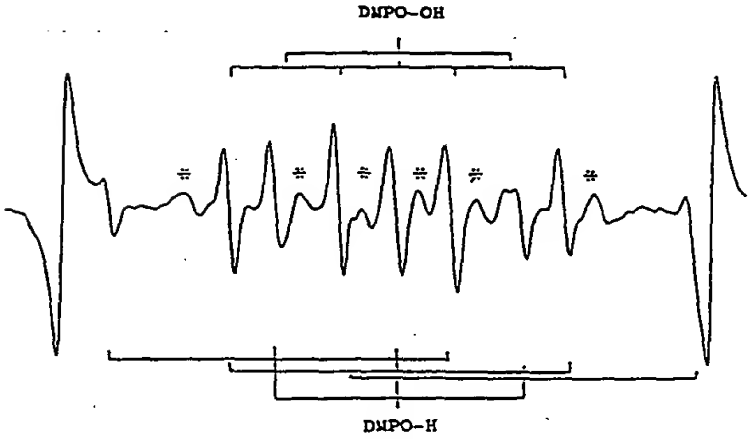

Fig. 5. ESR spectra of spin adducts of ·OH and ·H after mixing 75 μl 0.1 mM iron(II)–DETAPAC, 75 μl 0.01 mM H_2O_2, 15 μl, 0.92 M DMPO, in 50 μl pure water. The signal with "*" is the adduct of carbon center radical.

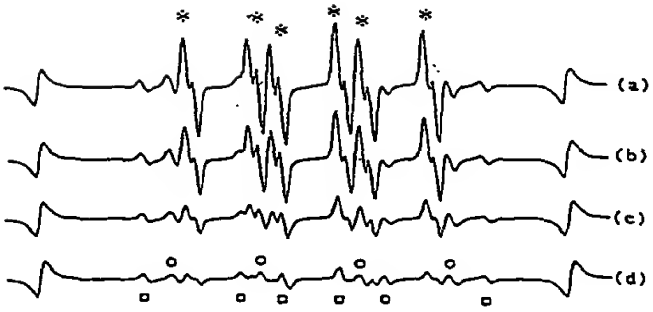

Fig. 6. ESR spectra observed in the reaction between 0.1 mM iron(II)–DETAPAC and ROOH of (a) 10 mM, (b) 5 mM, (c) 2.5 mM, (d) 1.25 mM ROOH concentration. The signal with "•" is the adduct of ROO·.

Table 1. Hyperfine Coupling Constants of Spin Adduct of Radicals, O_2^{-}, ·OH, ·H, R·, RO·, and ROO·[a]

Spin adduct.	a_N	a^{β}_H	a^{γ}_H
DMPO-O_2^-	1.41	1.14	0.13
DMPO-OH	1.48	1.48	
DMPO-H	1.64	2.25	
DMPO-OOR	1.45 (1.45)	1.05 (1.05)	0.14 (0.15)
DMPO-OR	1.49 (1.48)	1.57 (1.60)	
DMPO-R	1.64	2.24	

a) ( ) is reported values.

adducts (Fig. 7). In the case of the reaction with CAT, only RO· is observed (Fig. 8). The spin adducts of R·, RO·, and ROO· were identified using the hfcc values listed in Table 1.[29,30] As shown in Figs. 7 and 8, the spin adducts of ROO· and RO· changed, depending on each concentration of iron(II), CAT and ROOH.

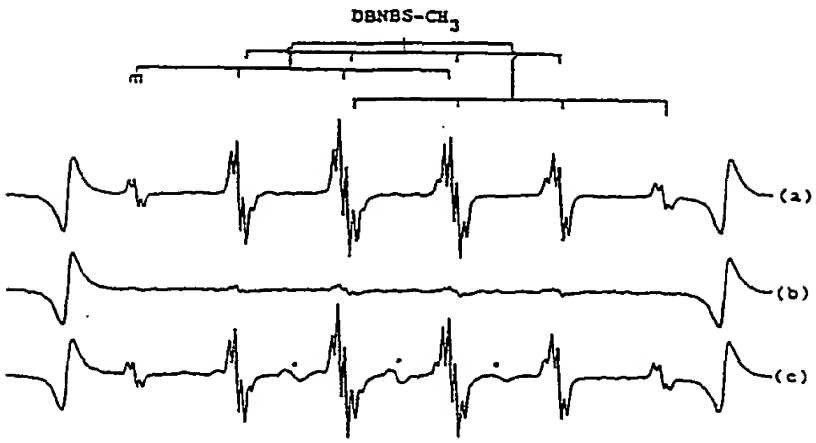

Fig. 7. ESR spectrum of $\cdot CH_3$ was observed by the use of DBNBS in the reaction between 1.0 mM iron(II) (a), iron(III) (b), iron(II)–DETAPAC (c) and 10 mM ROOH. The signal with "*" is the adduct of carbon center radical.

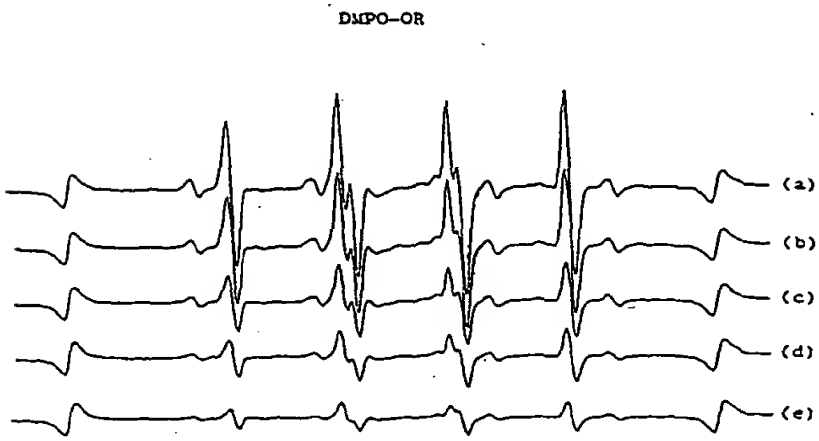

Fig. 8. ESR spectra observed in the reaction between ROOH and catalase of (a) 0.086 mM, (b) 0.043 mM, (c) 0.022 mM, (d) 0.011, (e) 0.006 mM catalase concentration.

In spin-trapping experiments, it can be confirmed that the results are very much affected by the concentration of DMPO used. For instance, if the DMPO concentration changed from 23 mM to 690 mM, the main observed spin adduct changed from RO· to ROO·, as well as the experiments for detecting ·OH and ·H. Thus, varying the DMPO concentration allows one to analyze specific radicals.

Reactivity of Water Soluble Antioxidants with $O_2^{\cdot-}$, ·OH, ROO·, and RO·. When DMPO was added to a solution of a radical-generating system, several spin adducts such as DMPO-O_2^-, DMPO-OH, DMPO-OOR, and DMPO-OR were detected. Various antioxidants such as L-ascorbic acid, D-isoascorbic acid, gallic acid, sorbic acid, and protocatechuic acid were diluted to 1 mM in pure water and added to a solution of the radical-generating system. Then, the amount of each spin adduct was found to decrease. These changes in the intensity of the ESR spectra were evaluated for the scavenging potencies of various antioxidants on $O_2^{\cdot-}$, ·OH, ROO·, and RO·. Table 2 shows the percent intensities of the spin adducts after the addition of antioxidants under the measurement conditions, as shown in the table, all of which were of equal concentration (1 mM). Based on Table 2, the scavenging action on $O_2^{\cdot-}$ of various antioxidants investigated decrease in the order D-isoascorbic acid > gallic acid > L-ascorbic acid > protocatechuic acid ≫ sorbic acid. As for ·OH, the potencies of antioxidants decrease in the order D-isoascorbic acid > L-ascorbic acid ≫ gallic acid = protocatechuic acid = sorbic acid=0%. For RO·, that order is gallic acid > protocatechuic acid ≫ sorbic

Table 2. Scavenging Activities(%) of Water Soluble Antioxidants L-Ascorbic Acid, D-Isoascorbic Acid, Sorbic Acid Protocatechuic Acid, and Gallic Acid against $O_2^{\cdot-}$, ·OH, ROO·, and RO·

Compound	$O_2^{\cdot-}$ a)	·OH b)	ROO· c)	RO· d)
L-Ascorbic acid	93.3	70.3	0	0
D-Isoascorbic acid	100.0	100.0	0	0
Sorbic acid	0	0	0	5.8
Protocatechuic acid	63.6	0	10.3	3.0
Gallic acid	96.5	0	0	28.5

Measurement conditions for generating $O_2^{\cdot-}$, ·OH, RO·, ROO· are shown as follows. a) $O_2^{\cdot-}$, 2 mM HPX+5.5 mM DETAPAC+0.4 U ml^{-1}+0.7 M DMPO. b) ·OH, 0.1 mM iron(II)–DETAPAC+1 mM H_2O_2+0.92 M DMPO. c) ROO·, 1.0 mM iron(II)–DETAPAC+5 mM ROOH+0.07 M DMPO. d) RO·, 1.0 mM iron(II)–DETAPAC+5 mM ROOH+0.07 M DMPO.

acid ≫ L-ascorbic acid = D-isoascorbic acid= 0%. In the case of ROO·, only protocatechuic acid show the function.

Reaction Mechanisms

The Fenton's Reaction. It has been reported that the formation of ·OH in a Fenton's reaction was directly confirmed using the spin trapping technique.[31,32] However, the reaction mechanism between iron(II) and H_2O_2 was assumed[33] to be

$$H_2O_2 + Fe^{2+} + H^+ \longrightarrow \cdot OH + Fe^{3+} + H_2O. \quad (1)$$

In our experiment two radical species (·OH and ·H) were observed in a Fenton's reaction used the H_2O_2 of low concentration for the concentration of iron(II). We thus propose the following reaction scheme, whereby both ·OH and ·H are generated:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+}\text{–}OOH^- + \cdot H \quad (2)$$
$$Fe^{3+}\text{–}OOH^- \longrightarrow Fe^{4+}{=}O^{2-} + \cdot OH \quad (3)$$
$$H_2O_2 + Fe^{2+} \longrightarrow \cdot H + \cdot OH + Fe^{4+}{=}O^{2-} \quad (4)$$

In addition, it can be speculated that the existence of ·OOH generates a reaction between O_2 and ·H. In the case of a high H_2O_2 concentration,[13] ·OH is mainly observed. We therefore propose the follow reaction:

$$H_2O_2 + Fe^{2+} \longrightarrow \cdot OH + Fe^{3+}\text{–}OH. \quad (5)$$

Catalase Reaction. As in the case of a Fenton's reaction, this catalase reaction mechanism with regard to the radical reaction has not been proven experimentally. The proposed catalase reaction mechanism is according to

$$\text{Catalase} + 2H_2O_2 \longrightarrow 2H_2O + O_2. \quad (6)$$

To clarify the mechanism for the oxidation of H_2O_2, the reaction of iron(II), iron(III), or ROOH with CAT and H_2O_2 were assayed under different conditions. Mixtures of just catalase and H_2O_2 gave no signals. However, the ESR spectrum of alkoxyl radical[$(CH_3)_3CO\cdot$] was detected in a reaction between the catalase and ROOH. We therefore propose the existence of the following reaction mechanism:

$$\text{Catalase} + (CH_3)_3COOH \longrightarrow (CH_3)_3CO\cdot + \text{catalase–OH} \quad (7)$$

$$2\ \text{Catalase} + H_2O_2 \longrightarrow 2\ \text{catalase–OH (Compound-II)}^{7)} \quad (8)$$

However, the generation of molecular O_2 in the reaction (Eq. 6) has been confirmed. Unknown factors in the mechanisms in the catalase reaction are as follows:

$$H_2O_2 \longrightarrow 2\cdot OH \quad (9)$$
$$H_2O_2 \longrightarrow \cdot H + \cdot OOH \quad (10)$$
$$\cdot OOH \longrightarrow \cdot H + O_2 \quad (11)$$
$$2\cdot H + 2\cdot OH \longrightarrow 2H_2O \quad (12)$$
$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (13)$$

The net equation (Eq. 13) is the same as that of Eq. 6.

Peroxides Reaction.[34] The reaction between iron(II) and ROOH generates three kinds of radicals (RO·, R·, and ROO·). Catalase reacts with ROOH, and generates RO·. Both results show that radical generation is modulated by the redox state and the conformation of the iron sites. The reaction of catalase is expressed as a function of the peroxidase.

These studies confirmed the previous results obtained from indirect assays, which indicated that $O_2^{\cdot-}$, ·OH, ·H, R·, RO·, and ROO· are generated by bio-relative reaction systems. Whether these compounds are the natural microbiological products of the oxidation process or simply intermediates in a complex series of reactions has yet to be determined. The methods employed here allow us to measure the antioxidant potencies of L-ascorbic, D-isoascorbic, sorbic, protocatechuic, and gallic acids on radical species such as $O_2^{\cdot-}$, ·OH, ROO·, and RO·.

References

1) R. J. Korthuis and D. N. Granger, in "Physiology of Oxygen Radicals," ed by A. E. Tayler, S. Matalon, and P. A. Warld, Williams & Wilkins, Baltimore MD (1986), p. 217—249.
2) M. G. Simic, D. S. Bergtold, and L. R. Karam, *Mutat. Res.*, 214, 3 (1989).
3) M. G. Simic, *Mutat. Res.*, 202, 377 (1988).
4) I. Fridovich, *Adv. Enzymol.*, 41, 35 (1974).
5) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 244, 6049 (1969).
6) N. Shimizu, K. Kobayashi, and K. Hayashi, *J. Biol. Chem.*, 259, 4414 (1984).
7) I. Yamazaki, *Protein, Nucleic Acid Enzyme*, 33, 2934 (1988).

8) E. Finkelstain, G. M. Rosen, E. J. Raukckman, and J. Paxton, *Mol. Pharmacol.*, 16, 676 (1979).
9) E. Finkelstain, G. M. Rosen, E. J. Raukckman, and J. Paxton, *Arch. Biochem. Biophys.*, 200, 1 (1980).
10) I. Ueno, M. Kohno, K. Mitsuta, Y. Mizuta, and S. Kanegasaki, *J. Biochem.*, 105, 905 (1989).
11) M. Makino, M. M. Mossoba, and P. Riese, *J. Am. Chem. Soc.*, 102, 4994 (1980).
12) M. Makino, M. M. Mossoba, and P. Riese, *J. Phys. Chem.*, 87, 1369 (1983).
13) M. Makino, M. M. Mossoba, and P. Riese, *FEBS Lett.*, 100, 23 (1979).
14) K. Mitsuta, Y. Mizuta, M. Kohno, H. Hiramatsu, and A. Mori, *Bull. Chem. Soc. Jpn.*, 63, 187 (1990).
15) I. Ueno, M. Kohno, K. Yoshihira, and I. Hirano, *J. Pharm. Dyn.*, 7, 563 (1984).
16) I. Ueno, M. Kohno, K. Yoshihira, and I. Hirano, *J. Pharm. Dyn.*, 7, 798 (1984).
17) H. Hiramatsu and M. Kohno, *JEOL News*, 23A, 7 (1987).
18) H. Miyagawa, T. Yoshikawa, T. Tanigawa, N. Yoshida, S. Sugino, M. Kondo, H. Nishikawa, and M. Kohno, *J. Clin. Biochem. Nutr.*, 5, 1 (1988).
19) T. Tanigawa, *J. Kyoto Pref. Univ. Med.*, 99, 133 (1990).
20) Y. Mizuta, K. Mitsuta, and M. Kohno, Proceeding of the 4th Biennial General Meeting of the Society for Free Radical Research, Kyoto, 1988, Abstr., pp. 9—13.
21) A. Mori, R. Edamatsu, M. Kohno, and S. Ohmori, *Neuroscience*, 15, 371 (1989).
22) S. Uchida, M. Hiramatsu, A. Mori, G. Monaka, I. Nishioka, M. Niwa, and M. Ozaki, *Med. Sci. Res.*, 15, 831 (1987).
23) F. Hayase, S. Hirashima, G. Okamoto, and H. Kato, *Agric Biol. Chem.*, 53, 3383 (1989).
24) T. Hatano, R. Edamatsu, M. Hiramatsu, A. Mori, Y. Fuzita, T. Yasuhara, T. Yoshida, and T. Okuda, *Chem. Pharm. Bull.*, 37, 2016 (1989).
25) E. G. Janzen, D. E. Nutter, and E. D. Davis, *Can. J. Chem.*, 56, 2237 (1978).
26) N. B. Nazhat, G. Yang, R. E. Allen, D. R. Blake, and P. Jones, *Biochem. Biophys. Res. Commun.*, 166, 807 (1990).
27) T. Ozawa and A. Hanaki, *Biochem. Biophys. Res. Commun.*, 136, 657 (1986).
28) A. Samuni, A. Samuni, and H. M. Swartz, *Free Radicals Med. Biol.*, 7, 37 (1989).
29) G. R. Buettner, *Free Radicals Med. Biol.*, 3, 259 (1987).
30) B. Kalyanaraman, C. Mottley, and R. P. Mason, *J. Biol. Chem.*, 258, 3855 (1983).
31) I. Yamazaki and L. H. Piette, *J. Biol. Chem.*, 265, 13589 (1990).
32) J. M. C. Gutteridge, L. Maidt, and L. Poyer, *Biochem. J.*, 269, 169 (1990).
33) C. C. Winterbourn, *Free Radicals Med. Biol.*, 3, 33 (1987).
34) R. A. Floyd, *Biochim. Biophys. Acta*, 756, 204 (1983).

Determination of Superoxide Dismutase Activity By Electron Spin Resonance Spectrometry Using the Spin Trap Method

Midori HIRAMATSU' and Masahiro KOHNO"

'Department of Neurochemistry, Institute for Neurobiology, Okayama University Medical School
"ESR Group, Analytical Instruments Engineering Department, JEOL LTD.

A novel method for O_2^- determination was established using electron spin resonance spectrometry with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) spin adduct of O_2^- generated by hypoxanthine and xanthine oxidase. A method for analyzing superoxide dismutase activity was developed using this method, and SOD activities in human plasma and the rat brain were measured.

It has been reported that active oxygen (O_2^-, ·OH, ·OOH, $^1O_2$, H_2O_2) may be related to inflammation, anti-cancer action, radiation damage, immunity, cataracts, hardening of arteries, heart ischemia and aging. In addition, this damage is thought to be derived from the degeneration of nucleic acids, enzymes and lipids by radical reaction. Superoxide dismutase (SOD; EC1.15.1.1.) is widely distributed in a living body. The superoxide anion is metabolized to hydrogen peroxide by SOD.[1] Recently, treatment of the above diseases with SOD derivatives is being investigated.

O_2^- and SOD activities have been analyzed using cytochrome C, nitroblue tetrazolium (NBT), epinephrine, pyrogaroll and ascorbic acid. These substances reduced by O_2^- were assayed by spectrophotometry. On the other hand, since the half-life of active oxygen is very short, it was very difficult to analyze active oxygen radicals by electron spin resonance (ESR) spectrometry. However, due to development of spin traps,[2] analyses of ·OH, ·OOH and O_2^- have become possible by ESR spectrometry using more stable spin adduct.[3,4]

First we tried to determine O_2^- as spin trap by ESR spectrometry using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) spin adduct generated by hypoxanthine (HPX) and xanthine oxidase (XOD). Then we established a method for determining SOD activity using the index of DMPO-O_2^- spin adduct and analyzed SOD activity in the rat brain and human plasma.

2-5-1 Shikata-cho, Okayama 700, Japan.

Experimental Method

Chemicals

DMPO, HPX, XOD, diethylenetriaminepentaacetic acid (DETAPAC) and SOD (bovine erythrocyte) were obtained from Sigma Chemical Company (St. Louis, Mo). All other chemicals and reagents were of the highest grade available from commercial suppliers.

Rat brain samples

Male Sprague-Dawley rats (250g) were killed by decapitation, and the whole brain was rapidly removed. The cortex, striatum, hippocampus, midbrain, hypothalamus

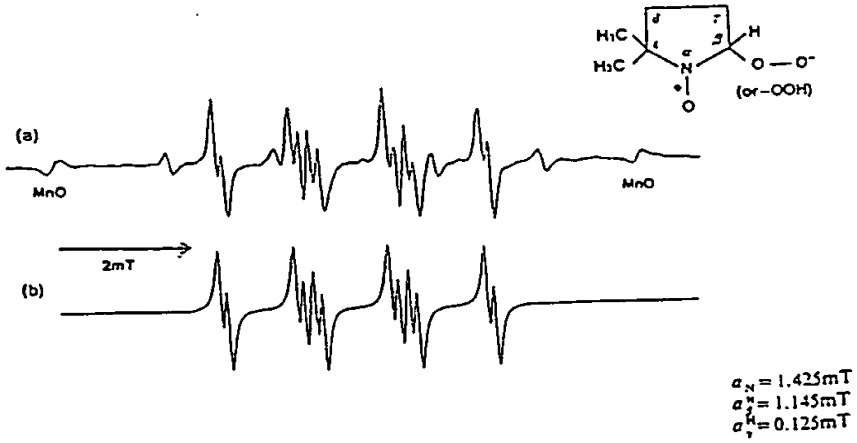

Fig. 1 (a) ESR spectrum of DMPO-O_2^- spin adduct. (b) Simulated ESR spectrum of Fig. 1 (a).

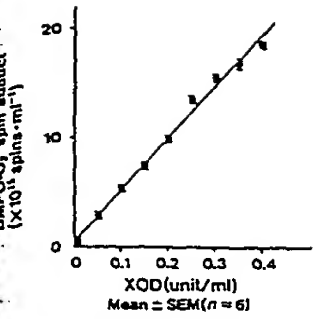

Fig. 2 The relationship between added concentration of XOD and formation of DMPO-O_2^- spin adduct.

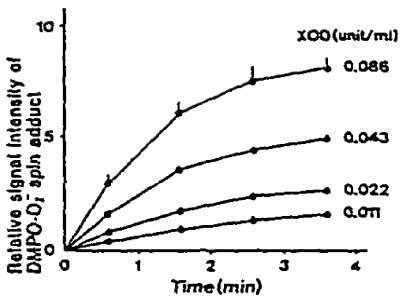

Fig. 3 Time course of formation of DMPO-O_2^- spin adduct after addition of xanthine oxidase (XOD).

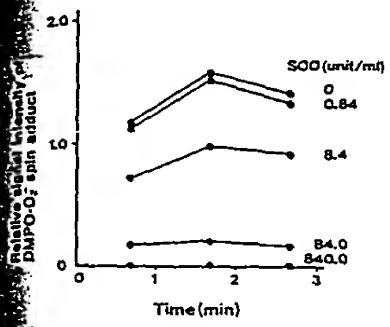

Fig. 4 Time course of formation of DMPO-O_2^- spin adduct after addition of superoxide dismutase (SOD).

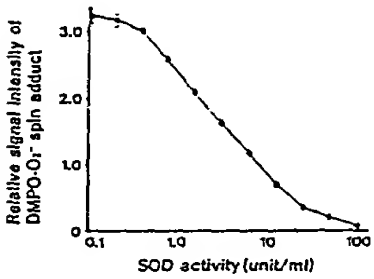

Fig. 5 Calibration curve of superoxide dismutase (SOD) activity.

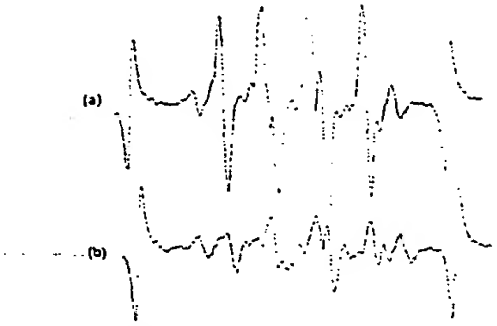

Fig. 6 ESR spectrum of DMPO-O_2^- spin adduct. (a) Hypoxanthine-xanthine oxidase system. (b) After addition of dialyzed SOD fraction of the rat cortex.

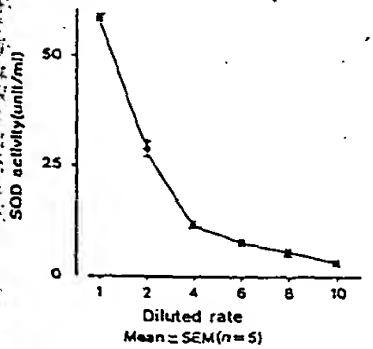

Fig. 7 SOD activity in dialyzed SOD fraction of the rat cortex with dilution.

pons and medulla oblongata and cerebellum were dissected on ice.[5] The tissue samples were homogenized in 9 to 45 volumes of ice-cold 0.1M sodium phosphate buffer (pH 7.8) and the homogenates were centrifuged at 105,000 × *g* for 60 min. The supernatants were dialyzed against 0.1M sodium phosphate buffer, pH 7.8 overnight at 4°C. The dialyzed SOD solution was kept at −80°C until analysis.

Human plasma samples

The subjects were 20 controls and 20 chronic hepatitis patients. These plasma were kept at −20°C until analysis.

Measurement of SOD activity

Fifty microliters of 2mM HPX, 35μℓ of 5.5mM DETAPAC, 50μℓ of the dialyzed SOD fraction or human plasma, 15μℓ of DMPO and 50μℓ of XOD (0.272 unit/mℓ) were put into a test tube and mixed by an automatic mixer. Then the solution was placed in a special flatt cell (volume 160μℓ, JEOL Ltd.) and DMPO-O_2^- spin adduct was analyzed by ESR spectrometry (JES-FE1XG).

Standard curve was made using 0.8 to 100 unit/mℓ of SOD, and manganese oxide was used as an internal standard. Calculation of spin number was carried out using the ratio of signal height intensity of 2,2-6,6-tetramethyl-4-hydroxyl-piperidine-1-oxyl (TEMPOL) having known spin quantities.

Conditions of ESR spectrometry for measurement of SOD activity were as follows: magnetic field, 335 ± 5mT; power, 8.0mw; response, 0.1sec; modulation, 0.2mT; temperature, room temperature; amplitude, 3.2×10^3; sweep time, 2min.

Results

Fig. 1 (a) shows the spectrum of DMPO-O_2^- spin adduct of O_2^- generated by the HPX-XOD system. Fig. 1 (b) shows the simulated ESR spectrum of the spectrum in Fig. 1 (a). The analyzed spectrum of DMPO-O_2^- spin adduct corresponded with the simulated spectrum. The quantity of DMPO-O_2^- spin adduct in Fig. 1 was about 6×10^{13} *spins/mℓ* (about 10^{-7}M).

Fig. 2 shows the relationsip between DMPO-O_2^- and XOD. DMPO-O_2^- spin adduct increased with the concentration of XOD in the range of 0 to 0.4 unit/mℓ. Fig. 3 shows the reaction time of DMPO-O_2^- spin adduct formation in the HPX-XOD system. The formation increased with the concentration of XOD, and it was saturated at 3.6 min at different concentration of XOD. Fig. 4 shows the reaction time of DMPO-O_2^- spin adduct formation with the addition of SOD to the HPX-XOD system. The spin adduct formation decreased with increasing concentrations of SOD. The formation was maximum at 1.4 min at different concentra-

Table 1 Effect of dialysis on SOD activity in the rat brain

	Cortex	Striatum	Cerebellum	Hippocampus	Midbrain	Pons-medulla oblongata	Hypothalamus
Dialysis (+)	24.7±1.8 (10)	31.6±4.2 (10)	11.7±1.0 (10)	8.3±0.9 (10)	18.4±2.7 (10)	12.4±2.2 (9)	24.8±4.6 (10)
Dialysis (−)	29.9±2.1 (10)	36.0±5.1 (10)	11.8±1.1 (10)	10.7±1.2 (10)	21.7±2.8 (10)	12.4±1.6 (9)	25.7±4.2 (10)

The results are expressed as unit/mg protein.
The values represent the mean ±SEM with experimental numbers in parentheses.

Table 2 SOD activity in human plasma

	Normal	Liver diseases
DMPO	5.20±1.32 (20)	3.52±1.50 (20)[a]
Nitrite	—	9.86±3.26 (20)[b]

The results are expressed as unit/ml protein. The values represnt the mean ± SD with experimental numbers in parentheses.
a: $p<0.005$ vs normal, b: $p<0.001$ vs DMPO by student's t-test.

tions of SOD. Fig. 5 shows standard curve of SOD activity. DMPO-O_2^- spin adduct of a fixed concentration decreased with the concentration of SOD. The precision was ± 5%.

The signal height of DMPO-O_2^- spin adduct decreased after addition of the dialyzed SOD fraction (Fig. 6). This show that this fraction has high SOD activity. SOD activity in the dialyzed SOD fraction from the rat cortex decreased with the dilution (Fig. 7). SOD activity in 7 areas of rat brain was 8.3 to 36.0 unit/mg protein. The activity was highest in the striatum follows in order by the cortex and hypothalamus. Dialysis had no significant effect.

SOD activity in human control plasma was 5.20±1.32 unit/mℓ. The activity in the plasma of chronic hepatitis patients was 3.52±1.50 unit/mℓ and the value was significantly lower than that in human controls (Table 1). The SOD activity in the patients' plasma determined by the method[6] using nitrite was about 60% higher than that using the method of ESR technique (Table 2).

Discussion

We described a method for determining O_2^- generated by the HPX-XOD system using ESR spectrometry with DMPO. The ESR spectrum of DMPO-O_2^- spin adduct by HPX-XOD corresponded with the simulated spectrum. Our ESR spectrogram (a_N, 1.43mT; a_β^H, 1.15mT; a_γ^H, 0.125mT) was similar to that of reported ESR parameter of DMPO-O_2^- spin adduct (a_N, 1.43mT; a_β^H, 1.17mT, a_γ^H, 0.125mT).[7] A spectrum with twelve peaks were identified with DMPO-O_2^- spin adduct.

The HPX concentration used for this experiment was in almost saturated state. For this reason, the formation of DMPO-O_2^- spin adduct was examined using different concentrations of XOD. The formation of DMPO-O_2^- spin adduct was found to be related to the concentation of XOD. K_m and V_{max} were 0.56 unit/mℓ and 32.26×10^{15} spins/mℓ respectively. In addition, the formation of DMPO-O_2^- spin adduct was saturated at 3.6 min and then the formation decreased. The formation was found to be diminished at about 8 min after XOD addition by the YT record.

These results showed that O_2^- could be measured by the ESR technique. Thus, we analyzed SOD activity as an index of DMPO-O_2^- spin adduct, and found that the formation of this adduct decreased with the concentration of added SOD and that SOD activity at a fixed concentration (12.5 unit/mℓ) decreased with concentration of XOD (0.088, 0.175, 0.350, 0.700, 1.400, 2.100, 2.800 unit/mℓ). These results showed that it was possible to measure SOD activity in the range of 0.39 to 25.0 unit/mℓ of a standard curve using Cu, Zn-SOD extracted from bovine erythrocytes.

The samples for the measurement of SOD activity were prepared according to Danh *et al.*[8] SOD activities in 7 areas of rat brain estimated by our method were found to be lower than the activity estimated by the NBT method (47.5 to 56.3 unit/mg protein), but the distribution pattern of SOD activity in the brain was the same. In addition, SOD activity in the human plasma estimated by ESR was lower than that estimated by the method using nitrite. These differences are thought to be derived from the different method used. As the methods of NBT and nitrite employ spectrophotometry, the values may be including background concentrations.

The new method for determination of SOD activity has the following advantages: (1) O_2^- as a substrate is adducted by DMPO in the correct rate, (2) the procedure is simple. Therefore, it allows reliable measurement of SOD activity in a shorter time compared with conventional methods.

References

1) McCord, J.M. and Fridovich, I. : Superoxide dismutase. *J. Biol. Chem.* 244, 6049-6055 (1969).
2) Janzen, E.G. :Spin trapping. *Accounts Chem. Res.*, 4, 31-40 (1971).
3) Harbour, J.R., Chow, V. and Bolton, J.R. : An electron spin resonance study of the spin adducts of OH and HO$_2$ radicals with nitrons in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. *Can. J. Chem.*, 52, 3549-3553 (1974).
4) Janzen, E.G., Nutter, D.E. and Davis, E.D. : On spin trapping hydroxyl and hydroperoxyl radicals. *Can. J. Chem.*, 56, 2237-2242 (1978).
5) Glowinski, J. and Iversen, L. : Regional studies of catecholamines in the rat brain — I. *J. Neurochem.*, 13, 655-669 (1966).
6) Oyanagni, Y. : Establishment of nitrite kit for SOD activity determination. *Medical Technology*, 4, 63-73 (1984).
7) Harbour, J.R. and Bolton, J.R. : Superoxide formation in spinach chloroplasts : electron spin resonance detection by spin trapping. *Biochem. Biophys. Res. Commun.*, 64, 803-809 (1975).
8) Danh, H.C., Benedetti, M.S. and Dostert, P. : Differential changes of superoxide dismutase activity in brain and liver of old rats and mice. *J. Neurochem.*, 40, 1003-1007 (1983).